JOURNAL OF VIROLOGY, May 1994, p. 2937–2946
0022-538X/94/$04.00+0

Vol. 68, No. 5

Human Cytomegalovirus Maturational Proteinase: Expression in *Escherichia coli*, Purification, and Enzymatic Characterization by Using Peptide Substrate Mimics of Natural Cleavage Sites

PHILIP J. BURCK,[1] DAVID H. BERG,[1] TAT P. LUK,[1] LORETTA M. SASSMANNSHAUSEN,[1] MARK WAKULCHIK,[1] DENNIS P. SMITH,[2] HANSEN M. HSIUNG,[2] GERALD W. BECKER,[2] WADE GIBSON,[3] AND ELCIRA C. VILLARREAL[1]*

Virology[1] and Biotechnology[2] Research Divisions, Lilly Research Laboratories, Eli Lilly & Company, Indianapolis, Indiana 46285, and Virology Laboratories, Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205[3]

Received 28 September 1993/Accepted 28 January 1994

The proteolytic processing of the human cytomegalovirus (HCMV) assembly protein, resulting in truncation of its C terminus, is an essential step in virion maturation. The proteinase responsible for this cleavage is the amino-terminal half of the protein encoded by the UL80a open reading frame. We have obtained high expression levels of this 256-amino-acid HCMV proteinase, assemblin, in *Escherichia coli*. In addition to the 28-kDa proteinase, a 15-kDa protein comprising the first 143 amino acids and a 13-kDa protein comprising the last 113 amino acids of the 28-kDa HCMV proteinase were present. Both the 28-kDa proteinase and the 15-kDa protein were purified by a two-step chromatographic procedure utilizing anion exchange in urea and dithiothreitol and size exclusion in NaSCN and dithiothreitol. Activation of the purified 28-kDa proteinase required denaturation in urea as well as complete reduction of all five cysteine residues in the molecule. Removal of the urea by dialysis with retention of the reducing agent yielded an active proteinase. Addition of glycerol to 50% enhanced the activity. The HCMV proteinase cleaved the peptides RGVVNASSRLAK and SYVKASVSPE, which are mimics of the maturational (M)- and release (R)-site sequences, respectively, in the UL80a-encoded protein. The cleavage site in the peptides was at the same Ala-Ser scissile bond as observed in the UL80a protein. The K_m value for the cleavage of RGVVNASSRLAK (M-site mimic) by the proteinase was similar to that for SYVKASVSPE (R-site mimic), but the turnover (k_{cat}) of the M-site peptide mimic substrate by the proteinase was six to eight times faster. The peptide homologs of the herpes simplex virus type 1 M- and R-site sequences in the UL26-encoded protein were also cleaved by the HCMV proteinase, although at rates slower than those for the HCMV substrates. The HCMV proteinase was inhibited by Zn^{2+} and by alkylating agents, but only at very high inhibitor concentrations. The purified 15-kDa protein, subjected to the same activation conditions as the 28-kDa proteinase, had no enzymatic activity against the HCMV M- and R-site peptide substrates.

Maturational proteinases have recently been described for cytomegalovirus (CMV) (1, 34) and for herpes simplex virus (HSV) (20, 27). These two enzymes, their homologs in other herpesviruses, and their substrate cleavage sites share important features that suggest they may be useful new targets for antiviral agents (34). These proteinases cleave a substrate, referred to here and elsewhere (34) as the assembly protein precursor (pAP), removing its carboxyl end (10, 20, 29, 34) by an endoproteolytic cleavage (20) and thereby forming the mature assembly protein (AP). The AP is an abundant phosphoprotein (2, 12, 18) found in immature intranuclear capsids but not in mature virions (9, 11). The AP is thought to be functionally analogous to the bacteriophage scaffolding protein (3, 18, 25) in facilitating capsid assembly, and its formation from pAP appears to be essential for the production of infectious virus (26). The site at which the pAP is cleaved, called the maturational cleavage site (M site), is conserved in all herpesviruses sequenced to date and has the consensus sequence (V/L)-X-A-S, where X is usually Gln, but can be Glu, Asn, or Asp (34). The gene encoding the pAP (~1 kb) is a member of a 3′-coterminal family of overlapping, in-frame genes (19, 32), the largest member of which (~2 kb) encodes the maturational proteinase (19, 20, 27, 34).

* Corresponding author. Phone: (317) 276-5160. Fax: (317) 276-1743.

The herpesvirus proteinase is autoproteolytic (1, 20, 34), and the full-length protein product of its gene (e.g., UL80a in human CMV [HCMV]) undergoes at least two cleavages, as indicated in the model previously presented (34). One of these cleavages is at its carboxyl end and corresponds to the pAP M-site cleavage because of the overlapping arrangement of their genes (19, 32). A second cleavage occurs near the midpoint of the molecule at a site called the release cleavage site (R site) (34). The R site was initially recognized by its similarity to the M site, but it is more highly conserved than the M site, contains an absolutely conserved Tyr at position 4 (P4) and has the consensus sequence Y-(V,L)-(K/Q)-A-(S/N), where P3 is usually Val and P1′ is usually Ser (34) (residues numbered according to reference 28). The proteolytic domain of the molecule has been localized to its amino half (1, 22, 34), which contains five highly conserved domains (CD1 to CD5 [31, 34]) that have been suggested to participate in the catalytic activity of the proteinase (31, 34). Although neither M- nor R-site cleavage is absolutely required for proteolytic activity (22, 31), R-site cleavage releases the mature form of the proteinase (i.e., assemblin) and may potentiate its activity.

In order to study the proteinase more readily in vitro, assays using proteins expressed in bacteria have been developed. Both the CMV and HSV proteinases have been expressed in *Escherichia coli* and shown to cleave their respective substrates in crude and partially purified systems (1, 5, 6). Results of these studies with *E. coli*-expressed proteins have been consistent with those obtained by using mammalian cells (20, 27, 31, 33, 34). More importantly, they have verified the fidelity of the bacterially expressed proteinases in cleaving at the biologically relevant R and M sites (1, 6). Additionally, work with the bacterially expressed CMV proteinase has revealed a third cleavage site, located between CD1 and CD2 in the enzymatic portion of that molecule, that has been suggested to be an inactivation mechanism (1) and has therefore been called the inactivation cleavage site (I site).

Inhibitor studies have shown that the activities of both the CMV and HSV enzymes are reduced somewhat by high concentrations of serine proteinase inhibitors (1, 21) and that the CMV proteinase is inhibited by high concentrations of zinc (1). Complementary studies using site-directed mutagenesis to identify catalytic site amino acids have implicated an absolutely conserved histidine (21, 31) and serine (31) in CD2 and CD3, respectively, as being essential for activity and have shown that an absolutely conserved cysteine in CD1 is not essential for enzymatic activity (22, 31). The sequence positions of these residues, however, do not align in an obvious way with other characterized proteinases, nor does the inhibitor profile correspond clearly with those of other characterized proteinases.

In this report we describe the purification of the 256-amino-acid, bacterially expressed HCMV proteinase, assemblin, and characterization of the purified enzyme with respect to (i) its activation and ability to cleave synthetic peptide mimics of the R and M sites of both HCMV and HSV type 1 (HSV-1) and (ii) its sensitivity to proteinase inhibitors. A 15-kDa fragment resulting from cleavage of assemblin at its I site has also been purified, characterized, and determined to be proteolytically inactive.

MATERIALS AND METHODS

Antisera. A peptide representing amino acids 2 through 20 (N′-TMDEQQSQAVAPVYVGGFL-C′) of the HCMV (strain AD169) proteinase was synthesized by American Peptide Company (Sunnyvale, Calif.). A carboxy-terminal cysteine was added to the peptide during synthesis to facilitate coupling to keyhole limpet hemocyanin (4). The coupled peptide in Freund's adjuvant was injected into rabbits subcutaneously and intramuscularly. Approximately 5 mg per rabbit was used in the primary injection, and a comparable amount was injected in each of two boosters 6 and 12 weeks later. Preimmune sera from the same animals showed no specific reactivity. The antiserum to the N-terminal peptide was designated α-HCPN2. Antiserum to a peptide representing the carboxy terminus (N′-KQLVGVTERESYVKA-C′) of the HCMV proteinase was prepared in the same manner and designated α-HCPC2.

Cells and virus. Human foreskin fibroblasts (HFF) were prepared, grown, and infected with HCMV strain AD169 as previously described (9).

Construction of the expression vector pHS563. A DNA fragment of UL80a encoding the first 256 amino acids was previously cloned into the eukaryotic expression vector pRSV.5 (neo) (23), resulting in a new plasmid, LM12 (33). Two oligonucleotide primers were designed to express the HCMV proteinase gene in *E. coli*. The two primers were used to amplify, by PCR (24), the HCMV proteinase gene with the plasmid LM12 (33) as a template. The oligonucleotides contained *Nde*I and *Bam*HI restriction sites, thereby introducing these two sites into the initiation and termination codon sequences, respectively, of the proteinase gene. The PCR-amplified proteinase gene was digested with the *Nde*I and *Bam*HI restriction enzymes, and the resulting DNA was purified by agarose gel (0.8%) electrophoresis. The purified proteinase gene was ligated to the *Nde*I-*Bam*HI-linearized *E. coli* expression vector pCZR332 (14). This resulted in the new plasmid pHS563. The ligation mixture was then used to transform *E. coli* K-12 RV308 (NRRLB-15624) (16), and the transformants were selected by their ability to attain log growth in the presence of tetracycline (10 μg/ml).

Expression of 28-kDa HCMV proteinase. *E. coli* cells containing the plasmid pHS563 were grown to stationary phase in TY medium (Difco Laboratories, Detroit, Mich.) containing tetracycline (10 μg/ml). The cells were then transferred to fresh medium and grown at 32°C until they reached a culture density (A_{550}) of 0.3 to 0.4. Production of the human 28-kDa HCMV proteinase in *E. coli* was induced by shifting the culture temperature to 42°C, and the cells were harvested after growing for 3 h in an air shaker incubator (New Brunswick, Edison, N.J.). The production of the HCMV proteinase was monitored by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis (SDS-PAGE), and the yield was determined by densitometric scanning of the protein gel.

Western blot (immunoblot) assays. Proteins were separated by SDS-PAGE, transferred electrophoretically to nitrocellulose (BA85; Schleicher & Schuell, Keene, N.H.), and blocked for 1 to 2 h at room temperature in 50 mM Tris (pH 7.4)–0.9% NaCl–5% Carnation dry milk. Antisera α-HCPN2 and α-HCPC2 were used as the primary antibodies at a 1:50 dilution in phosphate-buffered saline containing 0.1% Tween 20. Biotinylated goat anti-rabbit immunoglobulin G (BA-1000; Vector Laboratories, Burlingame, Calif.) at a concentration of 6 μg/ml was used as the secondary antibody. Bound antibody was detected by using the colorimetric Vectastain ABC immunoperoxidase kit (Vector Laboratories) (17).

Purification of HCMV proteinase and 15-kDa protein. All purification and activation procedures were performed at 5°C unless otherwise noted. *E. coli* cell paste was suspended in 0.05M Tris (pH 8.0) (8.0 ml/g of cell paste) and disrupted by sonication and overnight treatment with lysozyme (1.0 mg/g of cell paste). DE52 (2.0 g/g of cell paste) (Whatman Inc., Clifton, N.J.) was added to the broken-cell suspension to adsorb cellular debris and nucleic acid liberated by the cell disruption. After being stirred with the DE52 for 10 min, the suspension was vacuum filtered. The filtrate was centrifuged at 7,700 × *g* to sediment the inclusion bodies, which were then washed with 1% (vol/vol) acetic acid and distilled water. The washed inclusion bodies were then either used immediately or suspended in distilled water and stored at −80°C until they were used for purification of the HCMV proteinase and 15-kDa protein.

The washed inclusion bodies were dissolved in 0.05 M Tris–0.01 M dithiothreitol (DTT)–7 M urea, pH 8.0. The solution was centrifuged to produce a clear supernatant that was then applied to a column (3.2 by 19.7 cm) of Q Sepharose Fast Flow (Pharmacia LKB, Piscataway, N.J.) (500 mg of total protein per column run) equilibrated with 0.05 M Tris-HCl–0.01 M DTT–7 M urea, pH 8.0. The column was washed with this buffer, and the HCMV proteinase and 15-kDa protein were eluted with a discontinuous linear gradient of NaCl in the equilibration buffer at a flow rate of 5 ml/min. Elution conditions were as follows: equilibration buffer, 0 to 45 min; 0 to 0.1 M NaCl, 45 to 60 min; 0.1 to 0.12 M NaCl, 60 to 180 min; 0.12 to 1.0 M NaCl, 180 to 210 min. Fractions were collected every 5 min and assayed by SDS-PAGE in 14% gels (Novex, San Diego, Calif.). Appropriate pools of the 28-kDa proteinase and 15-kDa protein were made for further purification. Solutions of either the 28-kDa proteinase or 15-kDa protein were concentrated approximately 18-fold by ultrafiltration using stirred cells fitted with YM5 membranes (Amicon Div., W. R. Grace & Co., Danvers, Mass.).

The concentrated samples were applied, in a volume not exceeding 5% of the column volume, to a column (3.6 by 60 cm) of Superdex 75 (Pharmacia LKB) equilibrated with 0.05 M Tris-HCl–0.01 M DTT–2.0 M NaSCN, pH 8.0. Protein was eluted with the equilibration buffer at a flow rate of 3 ml/min, and fractions were assayed by SDS-PAGE. The pools of purified 28-kDa HCMV proteinase and 15-kDa protein were stored at $-80°C$ until needed.

Sequence analysis and mass spectrometry. N-terminal sequence analysis was performed by automated Edman degradation with an Applied Biosystems model 477A or model 470A sequencer. All mass spectra were obtained with a triple-quadrapole mass spectrometer fitted with an ion spray interface (model API III; PE-Sciex, Toronto, Canada). The inlet orifice potential was set at 50 V (+20 V relative to QO). Other parameters were adjusted as needed to obtain unit resolution over the mass range of 200 to 2,400 Da. The instrument was calibrated each day by using a mixture of polypropylene glycols supplied by the manufacturer. Data were acquired and analyzed with the manufacturer's software. Samples of the purified HCMV proteinase and the 15-kDa protein were prepared for analysis by desalting on a reversed-phase high-pressure liquid chromatography (HPLC) column (Aquapore RP-300, 0.46 by 25 cm; Applied Biosystems). The samples were applied to the column equilibrated in 0.1% trifluoroacetic acid (TFA). After the salts had passed through the column, the protein was eluted with 0.1% TFA–acetonitrile. Samples in this solvent were introduced into the mass spectrometer by direct infusion with a Harvard syringe pump at a flow rate of 1 to 5 μl/min.

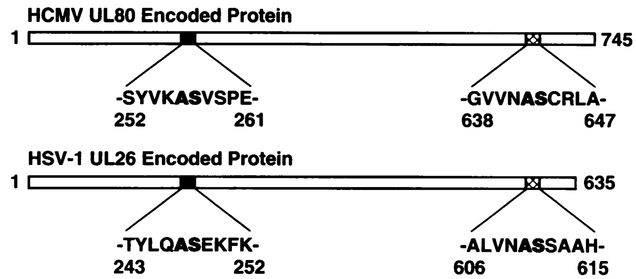
Activation of HCMV proteinase and 15-kDa protein. Purified samples of the enzyme from the Superdex 75 column were dialyzed against degassed and nitrogen-purged 0.1 M *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (HEPES)–0.2 mM EDTA–0.01 M DTT–7 M urea, pH 7.5. Urea was then removed by dialysis against 0.1 M HEPES–0.2 mM EDTA–0.01 M NaCl–2 mM DTT, pH 7.5. An equal volume of glycerol was added to the dialyzed enzyme, and the sample was allowed to stand overnight. The 15-kDa protein was treated in the same manner. Samples of activated HCMV proteinase and the 15-kDa protein were stored at $-20°C$ prior to use.

The number of free sulfhydryl groups in the activated, purified HCMV proteinase was determined by titration of the enzyme with Ellman's reagent [5,5'-dithio-bis(2-nitrobenzoic acid)] (Sigma Chemical Co., St. Louis, Mo.) (13). A sample of activated HCMV proteinase was exhaustively dialyzed against 0.05 M Tris, pH 8.0, containing 1 mM EDTA and 2% SDS under N_2 to remove exogenous DTT prior to titration with the Ellman's reagent. The protein concentration of the dialyzed solution was estimated by using an extinction coefficient of 0.992 $(mg/ml)^{-1}$ and a molecular weight of 28,041, with both values calculated from the sequence of the 256-amino-acid HCMV proteinase (Protein PP program [29a]).

The stability of the HCMV proteinase was determined by storing solutions of the enzyme for various lengths of time at -80, -20, and 37°C and determining activity with the HCMV M-site substrate.

Enzyme assays. HCMV proteinase activity was determined by monitoring the cleavage of the peptides SYVKASVSPE, a mimic of the R site, and RGVVNASSRLAK, a mimic of the M site (Fig. 1). All peptide substrates were greater than 95% pure and were obtained from American Peptide Company. HCMV proteinase (1.0 μM) was incubated with 120 μM substrate in 0.1 M 3-(*N*-morpholino)propanesulfonic acid (MOPS) (pH 7.2)–50% glycerol in a total volume of 30 μl for various lengths

A.



B.

		P5	P4	P3	P2	P1	P1'	P2'	P3'	P4'	P5'	
HCMV maturational site	R	G	V	V	N	**A**	**S**	S	R	L	A	K
HCMV release site		S	Y	V	K	**A**	**S**	V	S	P	E	
HSV-1 maturational site		A	L	V	N	**A**	**S**	S	A	A	H	
HSV-1 release site		T	Y	L	Q	**A**	**S**	E	K	F	K	

FIG. 1. (A) Schematic representation of the proteins encoded by the HCMV UL80a and HSV-1 UL26 open reading frames. The amino acid sequences (P5 to P5') around the R (solid bar) and M (cross-hatched bar) sites are shown. The nomenclature of Schechter and Berger (28) is used to denote the positions of the amino acids on either side of the scissile bonds. The amino acids at the scissile bond are in boldface. (B) Peptides used to assay the activity of the HCMV proteinase. The peptides mimic the sequences (P5 to P5') around the scissile bonds of the HCMV and HSV-1 R and M sites. The peptide mimic of the HCMV M site has an arginine residue added to the N terminus and a lysine added to the C terminus to increase the solubility of the peptide and to aid in derivatizing this peptide for automated assays. In addition, the cysteine at P2' in the native sequence is replaced by serine in the peptide mimic for ease of synthesis and handling.

of time at 37°C. The reaction was stopped by the addition of 120 μl of 0.1% TFA and assayed by reversed-phase HPLC (C-18, Zorbax RX-C18, 4.6 by 250 mm; Rockland Technologies, Chadds Ford, Pa.). The substrate and product fragments were resolved by gradient elution (5 to 20% acetonitrile, 10 min) in 0.1% (vol/vol in water) TFA at a flow rate of 1.0 ml/min. Activity was expressed as the percent loss of the substrate peak area.

Optimum conditions for enzymatic activity of the HCMV proteinase with the HCMV M- and R-site substrates were determined by varying the concentrations of glycerol from 0 to 50% (vol/vol) and the pH from 6.0 to 10.0. 2-(*N*-Morpholino) ethanesulfonic acid (MES), MOPS, HEPES, Tris, and piperidine hdrochloride buffers were used as appropriate in this pH range. When the optimum pH of and glycerol concentration in the assay buffer had been determined for the hydrolysis of the HCMV M-site peptide by HCMV proteinase, the concentrations of NaCl, EDTA, $CaCl_2$, and DTT in the assay buffer were also varied, as described in Results. Activity of the HCMV proteinase against analogous HSV-1 substrates (Fig. 1) was determined by cleavage of the peptide TYLQASEKFK, a mimic of the R site of the HSV-1 proteinase, and the peptide ALVNASSAAH, a mimic of the M site of the AP (6, 34).

Kinetic studies. Kinetic constants of the HCMV proteinase-catalyzed cleavage of the HCMV M- and R-site peptide substrates were determined by using the optimum assay conditions (0.1 M MOPS [pH 7.2], 50% glycerol) with the range of substrate concentrations outlined in Table 2. The initial time courses of the hydrolyses of the two peptide substrates were linear to the point of approximately 10 to 20% substrate turnover, so initial reaction rates were calculated only from data in this range. The appearance of the M- and R-site

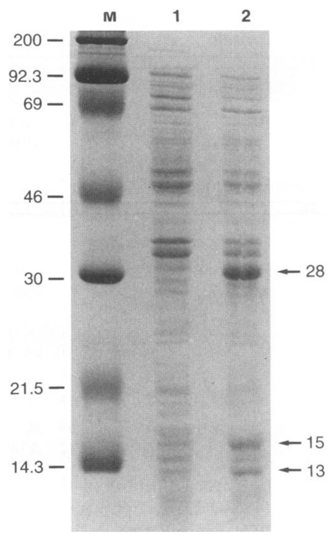


FIG. 2. Analyses of extracts from *E. coli* containing the pHS563 construct, showing a Coomassie brilliant blue-stained gel of whole-cell extracts of uninduced bacteria (lane 1) and of bacteria induced to express the 28-kDa proteinase (lane 2). Lane M shows stained molecular mass markers. Numbers at left indicate the molecular masses (in kilodaltons) of the corresponding markers, while numbers at right indicate the molecular masses of the corresponding stained bands.

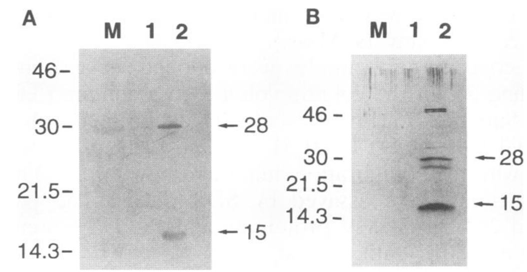


FIG. 3. (A) Analysis of whole-cell extracts from *E. coli* containing the pHS563 construct, showing Western blot SDS-PAGE of uninduced (lane 1) and induced (lane 2) whole bacterial cell extracts. The blots were probed with α-HCPN2 antiserum. Numbers at the left of each panel indicate the molecular masses (in kilodaltons) of the protein markers (lanes M), most of which are not visible since they do not react with the antiserum; numbers at the right of each panel indicate the molecular masses of the corresponding immunostained bands. (B) Analysis of HCMV-infected HFF, showing Western blot SDS-PAGE of an extract of mock-infected (lane 1) and HCMV-infected (lane 2) HFF.

hydrolysis products, SSRLAK and SYVKA, respectively, was quantitated by using a standard curve of concentration of peptide versus HPLC peak area. Disappearance of substrate for the M- and R-site peptide hydrolyses was determined in the same way by using a standard curve of RGVVNASSRLAK and SYVKASVSPE concentrations, respectively, versus HPLC peak area. Both substrate disappearance and product appearance were measured to verify that substrate disappearance was totally due to its enzymatic hydrolysis. The computer program GraFit (Erithacus Software by R. J. Leatherbarrow) was used for analysis of a Lineweaver-Burk graphical adaptation of the Michaelis-Menten equation.

RESULTS

Expression of HCMV proteinase (assemblin) in *E. coli*. The 256-amino-acid protein, identified as the HCMV proteinase released from the UL80a encoded protein (Fig. 1A) by R-site cleavage (1, 34), was expressed in *E. coli*. Production was monitored by SDS-PAGE analysis. The yield of HCMV assemblin (i.e., 28-kDa protein) was approximately 8% of the total cellular protein as determined by densitometric scanning of the dried SDS gel (Fig. 2, lane 2). New proteins of about 15 and 13 kDa, representing the amino and carboxyl portions, respectively, of the cleaved 28-kDa assemblin protein (see below), were also detected in the induced cells (Fig. 2, lane 2).

Western blot analysis using the α-HCPN2 antiserum showed the presence of a 28-kDa protein in the induced bacterial cells only (Fig. 3A, lane 2), demonstrating that the HCMV proteinase was being produced. The 15-kDa protein seen by Coomassie brilliant blue staining (Fig. 2, lane 2) also reacted with the α-HCPN2 antiserum, consistent with it being the amino half of the 28-kDa protein. Corresponding α-HCPN2-reactive 28- and 15-kDa proteins were also detected in HCMV-infected HFF (Fig. 3B, lane 2) (1) and in human cells transfected with a plasmid encoding HCMV assemblin (LM12 [33]) (13a), indicating that production of the 15-kDa protein is not an artifact of expressing the gene in bacteria. The infected cells also contain a 24-kDa protein (Fig. 3B, lane 2, immediately below the 28-kDa band) whose size is compatible with a cleavage of HCMV assemblin after Ala-209 in the sequence VDAS, which has similarity to the consensus cleavage site (1, 34). This 24-kDa band has not been observed in the induced bacterial cells (e.g., Fig. 3A, lane 2). The nature of the cross-reactive, HCMV-infected cell ~46-kDa band is presently not known.

Western blot analysis of whole-cell extracts and inclusion bodies from induced bacteria (Fig. 4) demonstrated that the 28-kDa protein was incorporated into inclusion bodies and reacted with antisera to either the amino (α-HCPN2) (Fig. 4A, lane 1) or carboxyl (α-HCPC2) (Fig. 4B, lane 1) end of the 28-kDa assemblin protein. The 15-kDa protein was also present in the inclusion bodies (Fig. 4A), as was the 13-kDa protein seen by Coomassie brilliant blue staining (Fig. 2, lane 2) and visualized here by the α-HCPC2 antiserum (Fig. 4B). The α-HCPC2 antiserum is directed to the carboxy terminus of

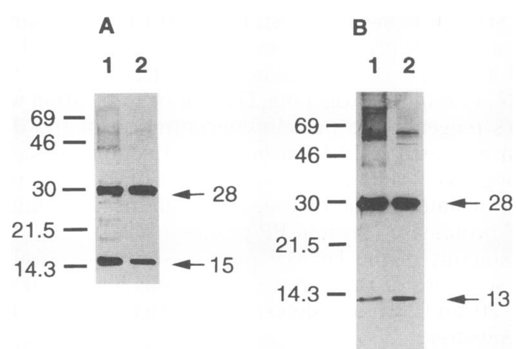


FIG. 4. Western blot analysis of inclusion granules and whole-cell extracts from bacteria containing the pHS563 construct. The blots were probed with the α-HCPN2 (A) or α-HCPC2 (B) antiserum. Lane 1 in each panel contained a 7 M urea solution of inclusion bodies, and lane 2 contained a whole-cell lysate of bacteria induced to express the HCMV 28-kDa proteinase. The molecular masses (in kilodaltons) of reference protein markers and of the immunoreactive bands are indicated, respectively, at the left and right of both panels.

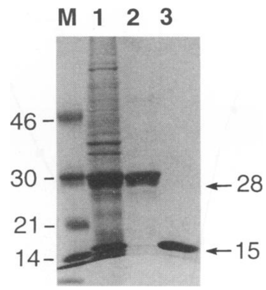


FIG. 5. Purification of the 28-kDa HCMV assemblin homolog and the 15-kDa protein as shown by SDS-PAGE (see Materials and Methods). Lane M, molecular mass markers; lane 1, 7 M urea solution of *E. coli* inclusion bodies; lane 2, purified HCMV proteinase; lane 3, purified 15-kDa protein. The gel was 14% polyacrylamide. Numbers at left indicate the molecular masses (in kilodaltons) of the Coomassie brilliant blue-stained protein markers; numbers at right indicate the molecular masses of the corresponding stained proteinase bands.

the 28-kDa proteinase, consistent with the 13-kDa protein being derived from the carboxyl half of the 28-kDa protein.

Purification of the HCMV proteinase and the 15-kDa protein. Initial purification of the 28-kDa HCMV proteinase and the 15-kDa protein was achieved by anion-exchange chromatography of a 7 M urea solution of the *E. coli* inclusion bodies on Q Sepharose Fast Flow. The proteinase peak was eluted with 0.1 M NaCl; the 15-kDa protein peak was eluted subsequently with 0.15 M NaCl. Final purification of both the 28-kDa HCMV proteinase (Fig. 5, lane 2) and the 15-kDa protein (Fig. 5, lane 3) was accomplished by size exclusion chromatography on Superdex 75 in the presence of the chaotropic agent sodium thiocyanate. The chaotrope was required to completely separate the 28-kDa HCMV proteinase and the 15-kDa protein from each other and from contaminating proteins, suggesting that they are capable of hydrophobic interaction. This procedure routinely yields preparations of the 28- and 15-kDa proteins that are at least 90 to 95% pure, as determined by protein staining following SDS-PAGE (Fig. 5).

Characterization of the HCMV proteinase and the 15-kDa protein. The purified 28-kDa HCMV proteinase was sequenced directly, and the mass was determined by electrospray mass spectrometry. The sequence of the first 10 N-terminal residues was MTMDEQQSQA, which is the N-terminal amino acid sequence for the 256-amino-acid protein, assemblin (Fig. 6), predicted from the DNA sequence of the HCMV proteinase gene. The mass spectrum showed at least two series of ions, one giving a mass of 28,041 Da, in agreement with the theoretical value for the 256-amino-acid HCMV proteinase, and the other giving a mass of 28,209 Da (data not shown). The identity of the latter species has not been determined; since no secondary sequence was observed during Edman degradation, it must either have the same N-terminal sequence as the 28,041-Da species or have a blocked amino terminus.

The 15-kDa protein was characterized by the same approach. Primary sequence analysis for eight cycles revealed the presence of two sequences, MTMDEQQS and TMDEQQSQ, which correspond to the N-terminal sequence of the proteinase with and without the N-terminal methionine. The amount of second sequence represented approximately 35% of that of the first. Electrospray mass spectrometry analysis of this sample gave the spectrum shown in Fig. 7A. The spectrum had a relatively high background intensity, but two distinct series of multiply charged protonated molecular ions could be seen. A second spectrum with a narrower scan range was obtained for the same sample (Fig. 7B). The ions at *m/z* 1100.0 and 1184.6, representing the protonated molecular ions with 14 and 13 charges, respectively, yielded a mass of 15,386 Da, and the ions at *m/z* 1109.4 and 1194.7 yielded a mass of 15,518 Da. The larger of these masses agreed with the sequence starting at amino acid 1 and running through amino acid 143; the smaller mass corresponded to the sequence starting with amino acid 2 and running through amino acid 143 of the HCMV proteinase.

Activities of HCMV assemblin and 15-kDa protein. HCMV proteinase, purified by the procedure described above, did not hydrolyze either the HCMV R-site or M-site peptide substrate. Furthermore, no enzymatic activity was detected when disulfide bond formation among the five cysteines of HCMV proteinase was promoted either by air oxidation (35) or by thiol redox pair interchange (15). Activity was observed, how-

```
                              CD2
1    MTMDEQQSQA  VAPVYVGGFL  ARYDQSPDEA  ELLLPRDVVE  HWLHAQGQGQ
51   PSLSVALPLN  INHDDTAVVG  HVAAMQSVRD  GLFCLGCVTS  PRFLEIVRRA
                   *                                CD3
101  SEKSELVSRG  PVSPLQPDKV  VEFLSGTYAG  LSLSSRRCDD  VEAATSLSGS
                                          *
                   CD1
151  ETTPFKHVAL  CSVGRRRGTL  AVYGRDPEWV  TQRFPDLTAA  DRDGLRAQWQ
201  RCGSTAVDAS  GDPFRSDSYG  LLGNSVDALY  IRERLPKLRY  DKQLVGVTER
251  ESYVKA

                 15-kDa                             13-kDa
     M1                               A143   A144                  A256
```

FIG. 6. Predicted amino acid sequence of the 256-amino-acid 28-kDa HCMV assemblin homolog. The five domains of conserved amino acids in the herpesvirus proteinases (CD1, F-155 to G-174; CD2, L-57 to V-72; CD3, V-121 to R-137; CD4, D-80 to P-91; CD5, P-13 to D-24) (31) are boxed. The histidine and serine residues in CD2 and CD3, respectively, which have been implicated as being essential for enzymatic activity (22, 31) are highlighted by shading and identified by an asterisk. The cleavage site at A-143–A-144, which results in the formation of the 15- and 13-kDa proteins, is indicated below the sequence in a straight-line depiction of the HCMV proteinase.

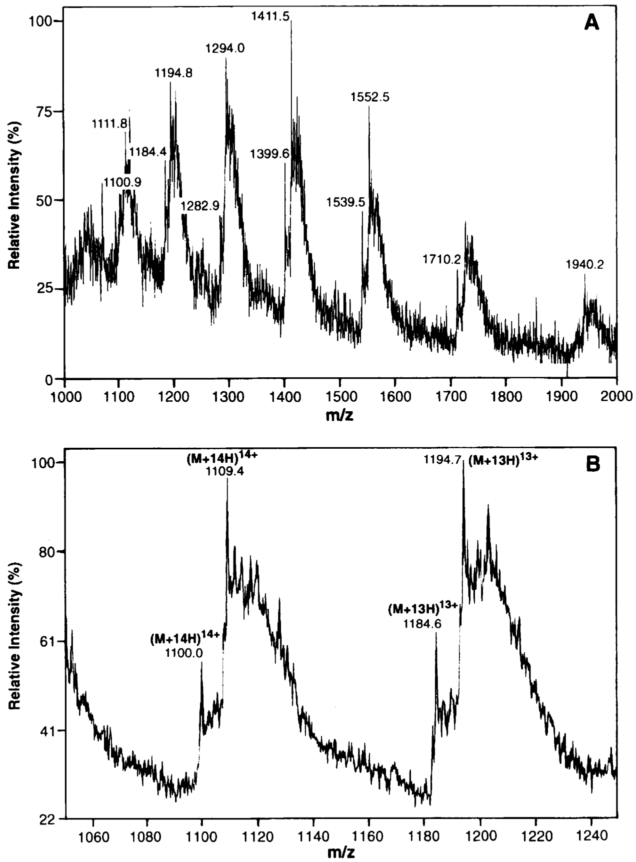


FIG. 7. Electrospray mass spectrum of the 15-kDa protein. The spectrum was obtained as described in Materials and Methods. (A) Survey spectrum from *m/z* 1000 to 2000. (B) Spectrum from *m/z* 1050 to 1250 obtained for the same sample. The ions used to calculate the masses are indicated.

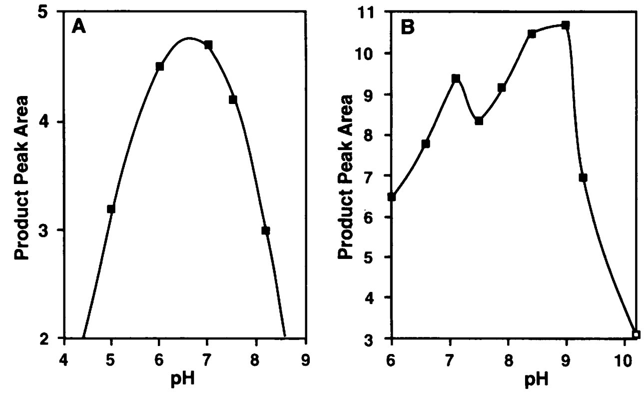


FIG. 8. pH dependence of HCMV proteinase-catalyzed hydrolysis of the HCMV R-site and M-site peptide mimics. The HCMV proteinase was incubated with the R-site substrate, SYVKASVSPE (A), and the M-site substrate, RGVVNASSRLAK (B), and the appearance of the products, SYVKA and SSRLAK, respectively, was determined as described in Materials and Methods. Fifty percent glycerol solutions buffered with 0.05 M MES (pH 6.0 and 6.8), HEPES (pH 7.3 and 7.7), Tris (pH 8.1, 8.6, and 9.2), and piperidine HCl (pH 9.5 and 10.4) were used for the activity determinations. Solid squares indicate actual data points; curved lines represent extrapolations of data points.

ever, when the enzyme first was dialyzed against 10 mM DTT in 7 M urea to reduce its cysteines and unfold it and then was allowed to refold by dialysis against degassed and N_2-purged buffers without urea but containing 2 mM DTT (see Materials and Methods). Recovery of enzymatic activity by this treatment suggested that all five of the cysteines in the functional proteinase exist in a reduced state. The absence of disulfide bonds in the active HCMV proteinase was confirmed by titration of the enzyme with Ellman's reagent. Three individual titrations indicated that the active HCMV proteinase contained 4.9 ± 0.5 mol of thiol per mol of enzyme, consistent with all five cysteines being in the reduced state.

The optimum pH for hydrolysis of the HCMV R-site peptide was approximately 7 (7.2 measured; 6.8 from extrapolated values) (Fig. 8A). Hydrolysis of the M-site substrate had a broader and apparently biphasic pH dependence, with optima of approximately 7 and 9 (Fig. 8B). This unusual pH dependence of the hydrolysis of the M-site substrate by the HCMV proteinase may result from protonation of the arginine and lysine residues at the termini of the substrate peptide. The activity of the proteinase against either of the substrate peptides was not grossly affected (i.e., $\leq$10%) by the following additions to the assay buffer: NaCl (0.1 to 0.9 M), EDTA (20 to 900 μM), $CaCl_2$ (10 to 900 μM), or DTT (0.1 to 0.9 mM). However, addition of glycerol (10, 20, 25, 40, and 50%) to the assay buffer increased the rate of substrate cleavage (1.4, 7.2, 8.1, 8.8, and 9.5-fold, respectively) relative to the rate with no glycerol. The effect of higher concentrations of glycerol on the proteinase activity was not investigated because the high viscosity interfered with accurate pipetting of these solutions. All subsequent cleavage assays were therefore done in 0.1 MOPS (pH 7.2)–50% glycerol.

When frozen at −20 or −80°C, as a 100- to 200-μg/ml solution in 50% glycerol, no loss of proteolytic activity was detected after 4 months. In contrast, the proteinase was stable at 37°C for only 1 h and then gradually lost ~40% activity by 24 h, probably because of autoproteolytic cleavage of the enzyme as determined by SDS-PAGE (data not shown).

It was established that cleavage of both the M- and R-site peptide mimics occurred at the expected Ala-Ser scissile bond (1, 34), by subjecting the substrate cleavage products to N-terminal sequence analysis. Cleavage of the M-site peptide mimic, RGVVNASSRLAK, yielded peaks P1 and P2 (Fig. 9A), whose sequences were determined to be SSRLAK and RGVVNA, respectively. Cleavage of the R-site peptide mimic yielded two peaks (P1 and P2) (Fig. 9B) whose sequences were SVSPE and SYVKA, respectively.

Kinetics. Rates of cleavage of the HCMV M- and R-site peptide mimics by HCMV assemblin indicated a much faster rate of cleavage of the M-site peptide. Over 95% of the M-site peptide was hydrolyzed in the first 30 min, but only 50% of the R-site substrate was cleaved in 2 h (Fig. 10A). Rates of cleavage of the corresponding HSV-1 substrates by the HCMV proteinase indicated a preference for HCMV-specific substrates (Fig. 10B). The HCMV M-site peptide was cleaved at an initial rate at least 10-fold greater than that for its HSV-1 homolog. Over 95% of the HCMV M-site peptide was cleaved in 30 min, whereas only 35% of the HSV-1 homolog was cleaved in 2 h. Hydrolysis of the HSV-1 R-site substrate was even slower, with only about 10% cleavage after 18 h (Fig. 10A). In the absence of added enzyme, there was no detected breakdown of the four peptide substrates following a 24-h incubation.

The K_m values for the hydrolysis of the M- and R-site

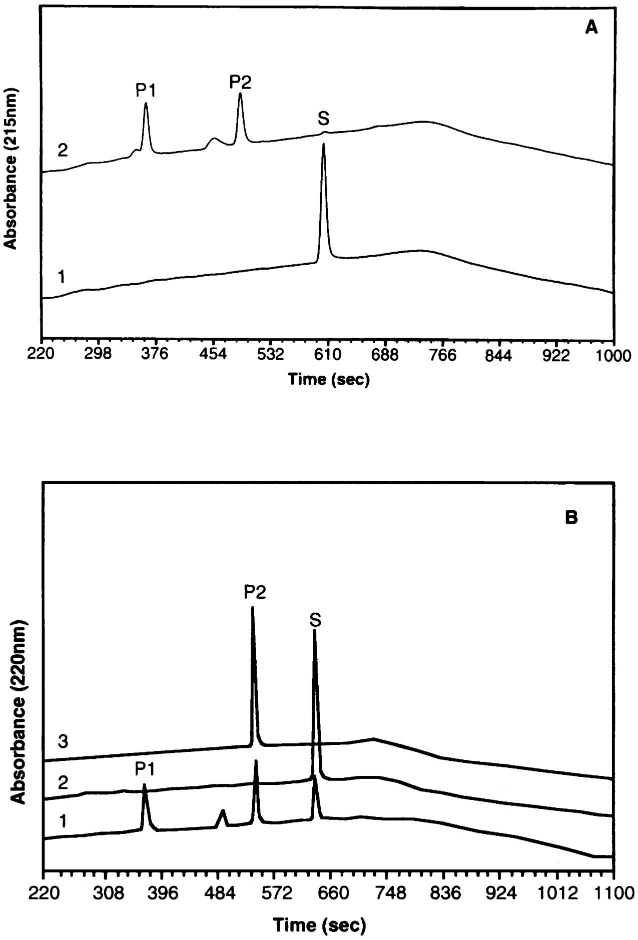


FIG. 9. HPLC analysis of hydrolysis of the HCMV M- and R-site peptides by HCMV proteinase. (A) The HCMV M-site peptide substrate, RGVVNASSRLAK, was incubated with the HCMV proteinase for 1 h at 37°C. The products of the hydrolysis were separated by reversed-phase HPLC as described in Materials and Methods. Elution profile 1 shows the substrate alone. Elution profile 2 shows the substrate following incubation with the proteinase; peaks P1 and P2 are products of the hydrolysis, and peak S is unhydrolyzed substrate. N-terminal sequence analysis demonstrated that the peptides in peaks P1 and P2 were SSRLAK and RGVVNA, respectively. (B) HPLC analysis of the hydrolysis of the HCMV R-site peptide substrate SYVKASVSPE by HCMV proteinase. The HCMV R-site substrate was incubated with the HCMV proteinase for 4 h at 37°C. The products of the hydrolysis were separated by reversed-phase HPLC as described in Materials and Methods. Elution profile 1 shows the substrate following incubation with the proteinase; peaks P1 and P2 are products of the hydrolysis, and peak S is unhydrolyzed substrate. Elution profile 2 shows the substrate alone. Elution profile 3 shows the product peptide, SYVKA, alone. N-terminal sequence analysis demonstrated that the peptides in peaks P1 and P2 were SVSPE and SYVKA, respectively. The small unlabelled peaks present in elution profiles 2 in panel A and 1 in panel B are not peptides but are artifacts due to the presence of reduced and oxidized DTT in the enzyme-containing samples applied to the HPLC column.

HCMV peptide substrates (Table 1) were similar when determined either by the disappearance of substrate or by the appearance of one of the peptide product peaks. However, the k_{cat} values for the hydrolysis of the M- and R-site substrates by the HCMV proteinase indicated a six- to eightfold-faster turnover of the M-site substrate by the enzyme.

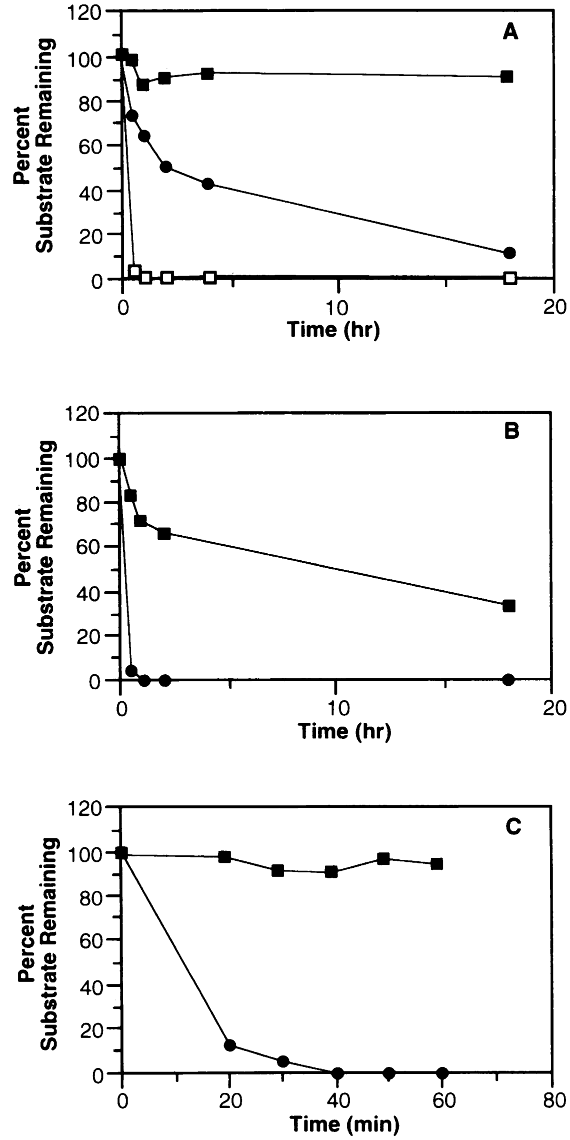


FIG. 10. Rates of hydrolysis of M- and R-site peptide substrates. The peptides were incubated with either the 28-kDa proteinase or the 15-kDa protein, and the disappearance of the substrate was determined as described in Materials and Methods. (A) Peptide substrates RGVVNASSRLAK (HCMV M site [□]), SYVKASVSPE (HCMV R site [●]), and TYLQASEKFK (HSV-1 R site [■]) incubated with HCMV proteinase. (B) M-site substrates RGVVNASSRLAK (HCMV [●]) and ALVNASSAAH (HSV-1 [■]) incubated with the HCMV proteinase. (C) M-site substrate RGVVNASSRLAK incubated with HCMV proteinase (●) and the 15-kDa protein (■).

The activity of HCMV proteinase was not inhibited by 100 or 250 μM solutions of phenylmethylsulfonyl fluoride (PMSF), *N*α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), benzamidine, cystatin, aprotinin, chymostatin, pepstatin A, leupeptin, or antipain. Some inhibition was observed when the HCMV proteinase was preincubated with 250 μM solutions of iodoacetamide, *N*-ethylmaleimide, diisopropylfluorophosphate, and *N*α-*p*-tosyl-L-phenylalanine chloromethylketone (TPCK); increasing concentrations of $ZnCl_2$ from 10 μM gave increasing inhibition of the enzyme, with complete inhibition at 500 μM (Table 2).

TABLE 1. Kinetic parameters for the hydrolysis of HCMV M-site and R-site substrates by HCMV proteinase, calculated from the initial velocities of the peptide cleavages

Peptide substrate (site)	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($μM^{-1}$ min^{-1})
RGVVNASSRLAK (M)	586[a]	16.0	0.027
	625[b]	12.4	0.023
SYVKASVSPE (R)	532[c]	1.9	0.0036
	450[b]	1.7	0.0038

[a] Calculated from the appearance of the product peptide, SSRLAK. The substrate concentration range used was 300 to 2,500 μM. The enzyme concentration was 0.2 μM.

[b] Calculated from the disappearance of the M- and R-site substrates. The substrate concentration range used was 391 to 626 μM. The enzyme concentration was 1.0 μM.

[c] Calculated from the appearance of the product peptide, SYVKA. The substrate concentration range used was 391 to 626 μM. The enzyme concentration was 1.0 μM.

Treatment of the 15-kDa protein by the same procedures that yielded an enzymatically active 28 kDa-HCMV proteinase did not render it enzymatically active in hydrolyzing either the HCMV M-site (Fig. 10C) or R-site (data not shown) peptide mimics.

DISCUSSION

We report here on (i) a procedure to produce large amounts of relatively pure and enzymatically active HCMV proteinase (assemblin), (ii) an in vitro assay procedure using the purified proteinase and peptide mimics that enables a quantitative evaluation of the M- and R-site cleavages, and (iii) use of the peptide cleavage assay to carry out an initial enzymatic characterization of the purified, native proteinase.

The gene encoding the 256-amino-acid HCMV proteinase, assemblin, has been expressed in *E. coli* at high levels, with the protein product accumulated as inclusion bodies. In addition to the expected 28-kDa proteinase, a 15-kDa fragment composed of residues 1 to 143 was found in the inclusion bodies in approximately equal amounts. A 13-kDa protein was also identified in the inclusion bodies by its reactivity with an antibody specific for the C terminus of the 28-kDa proteinase. This 13-kDa protein presumably represents the C-terminal fragment of the 28-kDa proteinase generated by cleavage at Ala-143, but amounts sufficient to confirm this were not isolated. Baum et al. (1) identified two proteins of about the same size (i.e., 16-kDa amino portion and 13-kDa carboxyl portion) in *E. coli* expressing the full-length HCMV proteinase gene, UL80a, but they concluded that the proteins arise from sequential cleavages of the 707-amino-acid assemblin precursor—first between Ala-193 and Ala-194 and then between Ala-256 and Ser-257—not directly from cleavage of the 256-amino-acid assemblin between Ala-143 and Ala-144. In contrast, our data are consistent with those from pulse-chase experiments done with CMV Colburn-transfected cells (31) and indicate that assemblin is not stable and does give rise, by direct cleavage (presumably in *trans*), to the smaller fragments.

Purification of the 28-kDa proteinase and the 15-kDa protein from *E. coli* inclusion bodies was accomplished by a two-step chromatographic procedure utilizing anion exchange in urea and DTT and size exclusion in NaSCN and DTT. This procedure yielded preparations of both proteins that were at least 90 to 95% free of other protein contaminants.

Activation of the 28-kDa proteinase prepared by this method required denaturation in urea as well as reduction of the cysteine residues. Removal of the urea by dialysis with retention of the reducing agent yielded an active proteinase with electrophoretic characteristics during SDS-PAGE that were essentially the same as those of the purified but not activated enzyme (data not shown). Promoting disulfide bond formation did not provide any detectable activation of the enzyme. Since the HCMV proteinase is an intracellular protein, this finding is consistent with previous observations that disulfide bonds occur predominantly in extracellular proteins (32). Attempts to activate the 15-kDa protein by the same procedures were unsuccessful. The lack of activity of the 15-kDa protein against either the HCMV M- or R-site peptide substrate confirms the prediction of Baum et al. (1) that the 16-kDa fragment produced by an A-143–A-144 cleavage in the HCMV proteinase precursor had no proteolytic activity.

Assays to measure the catalytic activity of HCMV proteinase were developed by using peptide substrates that mimic the amino acid sequences at the M and R sites of the UL80a-encoded protein and at homologous sites in the UL26-encoded HSV-1 protein (6, 34) (Fig. 1). The peptides were 10 or 12 residues long and included the highly conserved sequence observed in the P4 to P1′ positions of these cleavage sites (34). The HCMV M-site peptide was not an exact mimic in that the P2′ amino acid was changed from Cys to Ser to avoid possible spurious disulfide interactions during cleavage reactions, and the basic amino acids Arg and Lys were added to the N and C termini, respectively, of the peptide substrate to increase solubility and aid in derivatizing this peptide for automated assays.

Experiments to optimize the cleavage reaction showed that cleavages of both the M- and R-site peptide mimics had pH optima of approximately 7, close to that recently reported for an HSV-1 M-site peptide mimic cleaved by the HSV-1 assemblin homolog (7). It was also found that the cleavage reaction was dramatically enhanced by the addition of glycerol (8) but not notably affected by the presence of salt, calcium, EDTA, or DTT.

The specificity of the enzyme was studied by comparing substrate mimics of the cleavage sites of HCMV with one another and with those of HSV-1. Two points became evident: (i) HCMV peptide substrates were cleaved at a faster rate than the corresponding HSV-1 homologs, and (ii) the M-site peptides were cleaved at a faster rate than the R-site peptides. The faster cleavage rates of the HCMV peptide mimics compared with those of their HSV-1 homologs may reflect some preference of the HCMV proteinase for its own substrates; however,

TABLE 2. Effect of inhibitors on the activity of HCMV proteinase[a]

Inhibitor[b]	Concn (μM)	% Inhibition
$ZnCl_2$	1	0
	10	19
	100	54
	250	78
	500	100
DFP	250	28
TPCK	250	33
NEM	250	24
IAA	250	33

[a] Reactions were in 0.1 M MOPS (pH 7.2)–50% glycerol in a total volume of 30 μl. Enzyme (1.0 μM) was preincubated with the inhibitors at the final concentrations shown at 25°C for 1 h before addition of the substrate, RGV VNASSRLAK (120 μM). Hydrolysis of the substrate was measured 1 h later.

[b] DFP, diisopropyl fluorophosphate; IAA, iodoacetamide; NEM, *N*-ethylmaleimide. Benzamidine, cystatin, aprotinin, chymostatin, pepstatin A, leupeptin, antipain, TLCK, and PMSF were also tested at 100 and 250 μM, without effect. All inhibitors were purchased from Sigma Chemical Company.

the fact that the HSV-1 substrates were cleaved at all by the HCMV proteinase suggests at least some conservation of this function among the herpesvirus assemblin homologs. Because the amino acids in the P4 to P1′ positions of the respective HCMV and HSV-1 peptide substrates are very similar (Fig. 1), differences contributing to the observed substrate specificity may lie outside that four- to five-amino-acid consensus sequence. An alternate explanation for the observed difference in substrate specificity is that the assay conditions, determined by using the HCMV M-site peptide mimic, were less favorable for the HSV peptide.

The greater catalytic efficiency (k_{cat}/K_m) of HCMV assemblin for the HCMV M-site peptide substrate than for the R-site peptide substrate is not due to a dramatic difference in the K_m values of the two substrates but rather is due to a six- to eightfold difference in the turnover rate (k_{cat}) of the two substrates by the HCMV proteinase. The sequence similarity at the P3 to P1′ positions of the M and R cleavage sites, VNAS and VKAS, respectively, is consistent with a common substrate recognition site and, correspondingly, the similar K_m values for the two substrates. The higher turnover (k_{cat}) of the M-site substrate than of the R-site substrate may indicate that (i) the optimum recognition sequence for R-site cleavage is longer than the decapeptide we have used, (ii) the conserved Tyr at P4 in the R-site substrate in place of the smaller Val in the M-site substrate has a negative effect on the hydrolysis rates of the R-site substrates, (iii) an intramolecular versus an intermolecular cleavage mechanism at the R-site affects k_{cat}, (iv) there is an ordered cleavage of the UL80a-encoded protein in which one site must be cleaved before efficient cleavage of the other site can occur, or (v) the optimal cleavage conditions used for the peptide mimics in this study were significantly different.

Using in vitro-translated proteins and high inhibitor concentrations, Liu and Roizman (21) concluded that the UL26 proteinase of HSV-1 has a spectrum of inhibition suggestive of a serine proteinase. The findings by Welch et al. (31) that only one serine residue is absolutely conserved among the herpesvirus proteinases and that it is essential for proteolytic activity are also consistent with a serine nucleophile. It has been suggested, on the basis of inhibition of the HCMV proteinase in *E. coli* lysates by $ZnCl_2$, that this enzyme is a cysteine proteinase (1). Our studies with a panel of serine and cysteine proteinase inhibitors showed that both types could inhibit hydrolysis at high inhibitor concentrations. At these high inhibitor/enzyme ratios, it is not unlikely that diisopropylfluorophosphate, TPCK, *N*-ethylmaleimide, and iodoacetamide act as general alkylating agents rather than as specific active-site titrants of either a serine or a cysteine nucleophile. Although extremely high concentrations of $ZnCl_2$ were completely inhibitory, the observation that substitution of the only absolutely conserved cysteine (C-152 in HSV-1 and C-142 in simian CMV) in the proteinase does not abolish its activity in transfected cells (22, 31) argues against these herpesvirus maturational proteinases being cysteine proteinases.

The procedures reported here for preparing large amounts of the purified HCMV proteinase, assemblin, and quantifying its activity are expected to facilitate efforts to identify therapeutic agents for the treatment of herpesvirus infections by structure-based drug design and high-throughput inhibitor assays.

ACKNOWLEDGMENTS

We thank Travis Bennett and Melvin Johnson for expert technical assistance, Bruce McGathey and William Muth for fermentation of *E. coli* containing the pHS563 construct, Michele Smith for helpful comments on the preparation of the manuscript, and Carlos Lopez for his enthusiastic support of the project.

REFERENCES

1. **Baum, E. Z., G. A. Bebernitz, J. D. Hulmes, V. P. Muzithras, T. R. Jones, and Y. Gluzman.** 1993. Expression and analysis of the human cytomegalovirus UL80-encoded protease: identification of autoproteolytic sites. J. Virol. **67:**497–506.
2. **Braun, D. K., B. Roizman, and L. Pereira.** 1984. Characterization of posttranslational products of herpes simplex virus gene 35 proteins binding to the surfaces of full capsids but not empty capsids. J. Virol. **49:**142–153.
3. **Casjens, S., M. B. Adams, C. Hall, and J. King.** 1985. Assembly-controlled autogenous modulation of bacteriophage P22 scaffolding protein gene expression. J. Virol. **53:**174–179.
4. **Cox, M. A., J. Leahy, and J. M. Hardwick.** 1990. An enhancer within the divergent promoter of Epstein-Barr virus responds synergistically to the R and Z transactivators. J. Virol. **64:**313–321.
5. **Deckman, I. C., M. Hagen, and P. J. McCann III.** 1992. Herpes simplex virus type 1 protease expressed in *Escherichia coli* exhibits autoprocessing and specific cleavage of the ICP35 assembly protein. J. Virol. **66:**7362–7367.
6. **Dilanni, C. L., D. A. Drier, I. C. Deckman, P. J. McCann III, F. Liu, B. Roizman, R. J. Colonno, and M. G. Cordingley.** 1993. Identification of the herpes simplex virus-1 protease cleavage sites by direct sequence analysis of autoproteolytic cleavage products. J. Biol. Chem. **268:**2048–2050.
7. **Dilanni, C. L., C. Mapelli, D. A. Drier, J. Tsao, S. Natarajan, D. Riexinger, S. M. Festin, M. Bolgar, G. Yamanaka, S. P. Weinheimer, C. A. Meyers, R. J. Colonno, and M. G. Cordingley.** 1993. In vitro activity of the herpes simplex virus-1 protease with peptide substrates. J. Biol. Chem. **268:**25449–25454.
8. **Gekka, K., and S. N. Timasheff.** 1981. Mechanism of protein stabilization by glycerol: preferential hydration in glycerol-water mixtures. Biochemistry **20:**4667–4676.
9. **Gibson, W.** 1981. Structural and nonstructural proteins of strain Colburn cytomegaloviruses. Virology **111:**516–537.
10. **Gibson, W., A. I. Marcy, J. C. Comolli, and J. Lee.** 1990. Identification of precursor to cytomegalovirus capsid assembly protein and evidence that processing results in loss of its carboxy-terminal end. J. Virol. **64:**1241–1249.
11. **Gibson, W., and B. Roizman.** 1972. Proteins specified by herpes simplex virus. VIII. Characterization and composition of multiple capsid forms of subtypes 1 and 2. J. Virol. **10:**1044–1052.
12. **Gibson, W., and B. Roizman.** 1974. Protein specified by herpes simplex virus. Staining and radiolabeling properties of B capsids and virion proteins in polyacrylamide gels. J. Virol. **13:**155–165.
13. **Habeeb, A. F. S. A.** 1972. Reaction of protein sulfhydryl groups with Ellman's Reagent. Methods Enzymol. **25:**457–464.
13a. **Hall, ●., and W. Gibson.** Unpublished results.
14. **Heath, W. F., A. S. Cantrell, N. G. Main, and S. R. Jaskunas.** 1991. Mutations in the heparin-binding domain of human basic fibroblast growth factor alter its biological activity. Biochemistry **30:**5608–5615.
15. **Hober, S., G. Forsberg, G. Palm, M. Hartmanis, and B. Nilsson.** 1992. Disulfide exchange folding of insulin-like growth factor I. Biochemistry **31:**1749–1756.
16. **Hsiung, H. M., and W. C. MacKellar.** 1987. Expression of bovine growth hormone derivatives in *Escherichia coli* and the use of the derivatives to produce natural sequence growth hormone by cathepsin C cleavage. Methods Enzymol. **153:**390–401.
17. **Hsu, S. M., L. Raine, and H. Fanger.** 1981. A comparative study of the peroxidase-antiperoxidase method and an avidin-biotin complex method for studying polypeptide hormones with radioimmunoassay antibodies. Am. J. Clin. Pathol. **75:**734–738.
18. **Irmiere, A., and W. Gibson.** 1983. Isolation and characterization of a noninfectious virion-like particle released from cells infected with human strains of cytomegalovirus. Virology **130:**118–133.
19. **Liu, F., and B. Roizman.** 1990. The promoter, transcriptional unit, and coding sequence of herpes simplex virus type 1 family 35 proteins are contained within and in frame with the UL26 open reading frame. J. Virol. **65:**206–212.
20. **Liu, F., and B. Roizman.** 1991. The herpes simplex virus type 1

gene encoding a protease also contains within its coding domain the gene encoding the more abundant substrate. J. Virol. **65**:5149–5156.

21. **Liu, F., and B. Roizman.** 1992. Differentiation of multiple domains in the herpes simplex virus 1 protease encoded by the UL26 gene. Proc. Natl. Acad. Sci. USA **89**:2076–2080.
22. **Liu, F., and B. Roizman.** 1993. Characterization of the protease and other products of amino-terminus-proximal cleavage of the herpes simplex virus type 1 UL26 protein. J. Virol. **67**:1300–1309.
23. **Long, E. O., D. R. Rosen-Bronson, D. R. Karp, M. Malnati, R. P. Sekaly, and D. Jaraquemada.** 1991. Efficient cDNA expression vectors for stable and transient expression of HLA-DR in transfected fibroblasts and lymphoid cells. Hum. Immunol. **31**:229–235.
24. **Mullis, K. B., F. Faloona, S. Scharf, R. Saiki, G. Horn, and E. Erlich.** 1986. Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. Cold Spring Harbor Symp. Quant. Biol. **51**:263–273.
25. **Newcomb, W. W., and J. C. Brown.** 1991. Structure of the herpes simplex virus capsid: effects of extraction with guanidine hydrochloride and partial reconstitution of extracted capsids. J. Virol. **65**:613–620.
26. **Preston, V. G., J. A. V. Coates, and F. J. Rixon.** 1983. Identification and characterization of a herpes simplex virus gene product required for encapsidation of virus DNA. J. Virol. **45**:1056–1064.
27. **Preston, V. G., F. J. Rixon, I. M. McDougall, M. McGregor, and M. F. Al Kobaisi.** 1992. Processing of the herpes simplex virus assembly protein ICP35 near its carboxy terminal end requires the product of the whole of the UL26 reading frame. Virology **186**:87–98.
28. **Schechter, I., and A. Berger.** 1967. On the size of the active site in proteases. I. Papain. Biochem. Biophys. Res. Commun. **27**:157–162.
29. **Schenk, P., A. S. Woods, and W. Gibson.** 1991. The 45-kilodalton protein of cytomegalovirus (Colburn) B-capsids is an amino-terminal extension form of the assembly protein. J. Virol. **65**:1525–1529.

29a. **Shields, J. E.** Personal communication.

30. **Thornton, J. M.** 1981. Disulfide bridges in globular proteins. J. Mol. Biol. **151**:261–287.
31. **Welch, A. R., L. McNally, M. Hall, and W. Gibson.** 1993. Herpesvirus proteinase: site-directed mutagenesis used to study maturational, release, and inactivation cleavage sites of precursor and to identify a possible catalytic site serine and histidine. J. Virol. **67**:7360–7372.
32. **Welch, A. R., L. M. McNally, and W. Gibson.** 1991. Cytomegalovirus assembly protein nested gene family: four 3′-coterminal transcripts encode four in-frame, overlapping proteins. J. Virol. **65**:4091–4100.
33. **Welch, A. R., E. C. Villarreal, and W. Gibson.** Unpublished data.
34. **Welch, A. R., A. S. Woods, L. M. McNally, R. J. Cotter, and W. Gibson.** 1991. A herpesvirus maturational proteinase, assemblin: identification of its gene, putative active site omain, and cleavage site. Proc. Natl. Acad. Sci. USA **88**:10792–10796.
35. **White, F. H., Jr.** 1972. Reduction and reoxidation at disulfide bonds. Methods Enzymol. **25**:387–392.